# Flux analysis of central metabolic pathways in *Geobacter metallireducens* during reduction of soluble Fe(III)-NTA

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## Abstract

We analyzed the carbon fluxes in the central metabolism of *Geobacter metallireducens* strain GS-15 using $^{13}C$ isotopomer modeling. Acetate labeled in the 1$^{st}$ or 2$^{nd}$ position was the sole carbon source, and Fe-NTA was the sole terminal electron acceptor. The measured labeled acetate uptake rate was 21 mmol/gdw/h in the exponential growth phase. The resulting isotope labeling pattern of amino acids allowed an accurate determination of the *in vivo* global metabolic reaction rates (fluxes) through the central metabolic pathways using a computational isotopomer model. The tracer experiments showed that *G. metallireducens* contained complete biosynthesis pathways for essential metabolism, and this strain might also have an unusual isoleucine biosynthesis route (using acetyl-CoA and pyruvate as the precursors). The model indicated that over 90% of the acetate was completely oxidized to $CO_2$ via a complete tricarboxylic acid (TCA) cycle while reducing iron. Pyruvate carboxylase and phospho*enol*pyruvate carboxykinase were present under these conditions, but enzymes in the glyoxylate shunt and malic enzyme were absent. Gluconeogenesis and the pentose phosphate pathway were mainly employed for biosynthesis and accounted for less than 3% of total carbon consumption. The model also indicated surprisingly high reversibility in the reaction between oxoglutarate and succinate. This step operates close to the thermodynamic equilibrium possibly because succinate is synthesized via a transferase reaction, and the conversion of oxoglutarate to succinate is a rate limiting step for carbon metabolism. These findings enable a better understanding of the relationship between genome annotation and extant metabolic pathways in *G. metallireducens.*



Key words: labeled acetate, minimal medium, isoleucine, TCA cycle, acetyl-CoA transferase

## Introduction

Geobacter species have been known to be one of the dominant groups of microorganisms mediating iron reduction in the environment (21). They have been found to be ubiquitous in a myriad of subsurface environments. Detailed studies of their metabolism has revealed them to be capable of bioremediation of several heavy metals including uranium, plutonium, technitium, and vanadium as well as biodegradation of several organic contaminants including monoaromatic hydrocarbons (16, 17, 26). More recently, *Geobacter* species have been used to generate electricity from waste organic matter (2, 15, 19). These unique metabolisms make *Geobacter* species important players in the contaminated subsurface environment (18). *Geobacter metallireducens* was the first iron-reducing organism isolated that coupled complete oxidation of organic acids to reduction of iron oxides (20, 21). It completely oxidizes organic carbons such as fatty acids, alcohols, and monoaromatic compounds via the tricarboxylic acid (TCA) cycle (3, 20) coupled with the reduction of iron. The genomes of several *Geobacter* species have been sequenced, and proteome data are also available (5, 24). While the genome sequence and proteome are important for understanding *Geobacter*, they are not necessarily accurate representations of cell physiology and metabolism.

To quantitatively analyze central metabolism in *Geobacter sulfurreducens,* a constraint-based model was developed using the annotated genome sequence and a series of physicochemical constraints (thermodynamic directionality, enzymatic capacity and reaction stoichiometry) (22). While the model provided important insight into energy conservation, biosynthesis of building blocks (such as amino acids) and the relationship of the genotype to its phenotype, underdetermined models require one to assume an objective function (i.e., maximizing the specific growth rate) that may or may not be accurate and have difficulty

predicting fluxes through reversible reactions or reactions that may form futile cycles (7, 32, 39). Further, genes are often incorrectly annotated in sequenced genomes, and incorporation of these reactions into the model can affect the flux calculation. Even when properly annotated, the presence of a gene does not indicate if it is being expressed.

Here we report a different approach to analyze the fluxes in the central metabolic pathways of *Geobacter metallireducens* GS-15. The cells were fed [$^{13}$C]acetate, and the distribution of the $^{13}$C was measured in amino acids. Interpreted in the light of the genome annotation, a model based on the atom transitions between metabolites in biochemical reactions calculated the fluxes through the central metabolic pathway (12, 32, 33, 35). The model did not require energy balances for the calculation and resolved bidirectional or futile reactions. This study provided complementary flux information to the recent *in silico* model predictions and further extended our understanding of anaerobic carbon metabolism in *Geobacter* species.

## Materials and methods

**Growth conditions.** All media and solutions were prepared using strict anaerobic techniques. The standard *Geobacter metallireducens* bicarbonate-buffered freshwater medium was used (21) with one exception: one-tenth of the vitamin mix solution was used to minimize the isotopomer measurement "noise" introduced by unlabeled carbon from vitamins. Briefly, the medium was boiled under a $N_2$-$CO_2$ (80-20, vol/vol) headspace in order to remove the dissolved oxygen. It was then dispensed into anaerobic pressure tubes or serum bottles under a $N_2$-$CO_2$ (80-20, vol/vol) headspace. The anaerobic pressure tubes or serum bottles were capped with thick butyl-rubber stoppers and sterilized. [1-$^{13}$C] Sodium acetate and [2-$^{13}$C] sodium acetate (both of 99% purity) were obtained from Cambridge Isotope Laboratories Inc (Andover, Massachusetts). Anoxic aqueous stock solutions were prepared of Ferric-NTA (1M), [1-$^{13}$C] or

[2-$^{13}$C] sodium acetate (1 M) under a headspace of $N_2$-$CO_2$. These stocks were delivered anaerobically into culture tubes and serum bottles via a needle. *G. metallireducens* GS-15 was routinely cultured on anaerobic basal medium (21) using 5 mM acetate and 15 mM Fe-NTA as the electron donor and acceptor, respectively, under a $N_2$-$CO_2$ (80:20, vol:vol) headspace. A 10% inoculum from the unlabeled stock culture was made into the [1-$^{13}$C] or [2-$^{13}$C] acetate medium containing equivalent amounts of electron donor and acceptor. After growth reached the mid-log phase, cells were transferred again into the same labeled medium to minimize the effect of unlabeled carbon from the initial inoculum. This sub-culture protocol was repeated twice. All incubations were performed at 30°C.

**Determining metabolite concentrations and biomass composition.** The standard ferrozine assay was used to measure Fe(II) concentration during growth on acetate and Fe-NTA (21). Cell counts were performed using a microscope and acridine orange to stain cells. Briefly, a 100-µl sample was added to 900-µl, 0.1% sodium polyphosphate solution and mixed well. Ten µl of this cell suspension was pipetted onto a 6 mm well of slide. The slide was dried and heat fixed. Twenty-five (25) µl of acridine orange stain was used to stain the wells containing several dilutions of the cell samples. The slides were incubated in the dark for 2 minutes, washed, and then dried; the cells were counted using fluorescent microscopy. The concentrations of acetate in the culture supernatant (following centrifugation of the culture at 10,000 × g for 20 minutes at 4°C) were measured using enzyme assays (r-Biopharm, Darmstadt, German). The amino acid composition of the biomass protein was quantified using the Beckman 6300 amino acid analyzer (Beckman Coulter, California), performed by the Molecular Structure Facility at the University of California, Davis (http://msf.ucdavis.edu). Biomass constituents were taken from the literature: protein (46%), RNA (10%), DNA (4%), lipids (15%), total carbohydrate (15%),

lipopolysaccharides (4%), and peptidoglycan (4%) (22), and those data were the initial estimates for the isotopomer model to calculate fluxes into biomass.

**Isotopomer analysis of protein amino acids by GC-MS** (33-35)**.** A 200-mL cell culture ($2\times10^8$ cells/ml) was harvested by centrifugation at 10,000 × g for 20 minutes at 4°C and sonicated subsequently for 3 minutes. The protein from the resulting lysate was precipitated using trichloroacetic acid and then hydrolyzed in 6 M HCl at 100°C for 24 hours. The amino acid/HCl solution was dried under nitrogen flow overnight. GC-MS samples were prepared in 100 µl tetrahydrofuran (THF) and 100 µl N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich, USA). These samples were derivatized at 70°C for 1 hour, producing tert-butyldimethlysilyl (TBDMS) derivatives (33-35). One µL of the derivatized sample was injected into a gas chromatograph (Agilent, model HP6890) equipped with a DB5-MS column (J&W Scientific, Falsom CA) and analyzed using a mass spectrometer (Agilent, model 5973). The GC column was held at 150°C for 2 minutes, heated at 3°C per minute to 280°C, heated at 20°C per minute to 300°C, and held for 5 minutes at that temperature.

**Annotated pathway map and algorithm for flux calculation**. The central biochemical pathways in *Geobacter metallireducens* GS-15 include gluconeogenesis, the TCA cycle, and the pentose phosphate (PP) pathway (1). Each reaction and its corresponding gene are listed in Supplementary Table S-1. To reduce computational time, the fluxes through the pools of amino acids, carbohydrate, and RNA/DNA were loosely constrained by the biomass production and the measured average biomass composition (Supplementary Table S-2), and those fluxes were optimized using the isotopomer model based on the amino acid label information. The reversible reactions were characterized by their net flux, $v_i$, and their exchange flux, $v_i^{exch}$. The net flux was defined as the difference between forward and backward fluxes, ($v_i^{\rightarrow}$- $v_i^{\leftarrow}$). The exchange flux,

$v_i^{exch}$, was the smaller of the forward and backward fluxes, min($v_i^{\rightarrow}$, $v_i^{\leftarrow}$), and was used to calculate the exchange coefficient, $exch_i$, according to (33, 38):

$$v_i^{exch} = \frac{exch_i}{1 - exch_i} \tag{1}$$

Exchange coefficients for all reactions were searched in the range [0 1] (41). Since atom transitions between metabolites in the biochemical network were known and the path that each atom took through the network could be traced using the model, the steady-state isotopomer distributions in the intracellular metabolite pools for a given flux distribution were obtained via the isotopomer mapping matrices (29, 32) (using MATLAB 6.0, Mathworks, USA); these isotopomer distributions were used to simulate MS data (m/z=M0, M1, M2…). Our isotopomer model, like most non-linear systems, did not possess a simple analytic solution. The final solution in our isotopomer model was searched based on an objective function defined as:

$$\varepsilon(v_n) = \sum_{i=1}^{a} \left( \frac{M_i - N_i(v_n)}{\delta_i} \right)^2 \tag{2}$$

where $v_n$ were the unknown fluxes to be optimized in the model program, $M_i$ were the measured MS data when [1-$^{13}$C] or [2-$^{13}$C] acetate was used as the carbon source, respectively; $\delta_i$ were the corresponding measurement errors; $N_i$ were the corresponding model-simulated MS data when a complete set of flux distribution $v_n$ and exchange coefficients $exch_i$ were given to the isotopomer model. The optimal fluxes were calculated to be such that ε was minimized using a simulated annealing approach with different initial conditions (27, 33). The initial annealing temperature was set to 50 and the final one to 0.01, with the temperature being decreased 100 times by a set fraction each time. In each run, 10,000~100,000 moves were used, and the algorithm was restarted from the final position several times to check the reliability of the minimum. The examples of MATLAB programs for calculation of flux and exchange coefficients are available

at http://vimss.lbl.gov/DvHFlux/AdvancedCodesWithAMM_IMM.rar. The solution produced isotopomer predictions consistent with measured data from both [1-$^{13}$C] and [2-$^{13}$C] acetate experiments.

## Result and discussion

***Geobacter metallireducens* GS-15 growth kinetics in minimal medium.** GS-15 grew in minimal medium and completely oxidized acetate as the sole carbon and energy source by reducing $Fe^{3+}$ to $Fe^{2+}$ (Figure 1). The doubling time was ~5 hours with a late mid-log phase density of ~$2.3\times10^8$ cells/ml, and the corresponding biomass concentration was 4.8±0.3 mg/L with a yield of 3.2 gdw/mol acetate. In the final sampling point, about 1.5 mM acetate was consumed and 11 mM $Fe^{2+}$ was generated (equivalent to dissimilating 1.4 mM acetate). This result indicates that the Geobacter's biomass yield from oxidization of acetate is three times lower than the thermodynamic yield predictions (16.8 gdw/mol acetate) (36, 40). At standard conditions (1 atm and 25ºC indicated by the Θ superscript), $Fe^{3+}$ ($\Delta G^{\Theta}$=-24.38 kcal/eq) has a similar electron potential as oxygen ($\Delta G^{\Theta}$=-25.28 kcal/eq) (23). However, *Geobacter*'s Fe(III) reduction site is extracellular (not in the cytoplasm); under $Fe^{3+}$-NTA reduction, the electrons have to be transported outside of the cell or into the periplasmic cytochrome pool, but the protons remain in the cytoplasm (22). This could result in dissipating the membrane potential and acidifying the cytoplasm, which in turn could reduce the biomass yield that results from acetate via $Fe^{3+}$ reduction compared to that obtained during oxygen or fumarate reduction (9, 22).

***Isotopomer analysis of labeling pattern in protein amino acids by GC-MS.*** Labeled acetate (1$^{st}$ position or 2$^{nd}$ position) was used in independent experiments. GS-15 was harvested in the exponential growth phase from each batch culture (a quasi steady-state that the cells are

under a balanced growth condition) (10, 28, 31). Two types of positively charged amino acid species from the biomass protein were clearly observed by GC-MS: unfragmented amino acids [M-57] and fragmented species [M-159] that had lost the α-carboxyl group (4, 6, 14, 37). The natural abundance of heavy isotopes common in organic molecules as well as the derivatization agents was corrected for by using published algorithms (37). The corrected GC-MS data for eight key amino acids useful for model calculation including $[M-57]^+$ and $[M-159]^+$ are provided (Table 1). The isotopomer distributions in the amino acids from hydrolyzed protein were used to examine the metabolic pathways. For example, the different labeling patterns of alanine and serine indicate that their precursors were not same; i.e., alanine is derived from pyruvate, while serine is derived from phosphoglyceric acid (PGA). In each type of experiment, isotopomer patterns in some amino acids from the same precursor were similar and provided redundant isotopomer information (11): i.e., threonine and aspartate from oxaloacetate, tyrosine and phenylalanine from phospho*enol*pyruvate and erythrose-4-phosphate. Therefore, only one amino acid from each precursor listed in the table was used for model calculations. GC-MS cannot accurately measure the ion fragment $[M-57]^+$ (no loss, m/z=302) for leucine and isoleucine because of the overlay of mass peaks (mass fragment with only the α and β carbons of leucine/isoleucine also has an m/z of 302) (37).

***Determination of the flux distribution using the isotopomer model.*** The published annotated genome sequence of *Geobacter metallireducens* indicates that several amino acid biosynthesis pathways (e.g., lysine, valine, leucine, isoleucine, and alanine) are incomplete (1). However, *Geobacter metallireducens* is able to grow in minimal medium with acetate as its sole carbon source, and therefore must contain complete energy and biosynthesis pathways for essential metabolites, i.e., *G. metallireducens* may have undocumented genes encoding some

amino acid biosynthesis enzymes. The $^{13}C$ flux analysis can aid annotation of un-annotated pathways. The isotopomer model calculation from two tracer experiments (with [1-$^{13}C$]acetate and [2-$^{13}C$]acetate) gave similar flux distribution results (Figure 2). The predicted labeling patterns of all metabolites (except isoleucine), based on calculated fluxes and exchange coefficients, matched relatively well the measured data (deviations are within the noise from triplicate tracer experiments), and this indicates that model calculations are of good quality (Figure 3). The discrepancy between the measured isoleucine $[M159]^+$ data and the model predictions indicates that isoleucine is not synthesized from oxaloacetate and pyruvate (Table 1). This is consistent with the fact that a key step in the usual isoleucine biosynthesis pathway (EC 4.2.1.9, dihydroxy-acid dehydratase) in G. *metallireducens* is missing, and an alternative isoleucine biosynthesis pathway may be present. The isotopomer labeling of isoleucine was identical to that of leucine from both tracer experiments, and this indicates that isoleucine shares the same precursors (i.e., pyruvate and acetyl-CoA) as leucine. This hypothesis is supported by recent discovery of citramalate synthase in *Geobacter sulfurreducens*; thus, isoleucine is likely derived from acetyl-CoA and pyruvate via citramalate as an intermediate in *Geobacter* species (8) (personal communication with M. Coppi, University of Massachusetts, Amherst, MA and S. Van Dien, Genomatica, San Diego, CA).

The conversion of acetate to acetyl-CoA (acetate uptake rate of 21±1.6 mmol/gdw/h, assumed to be 100 in the model calculation) may be catalyzed by two independent enzymes (acetyl-CoA transferase or acetate kinase) (Figure 2). The acetyl-CoA produced branched into three pathways: the major flow (19 mmol/gdw/h, relative flux v = ~90) was into a complete TCA cycle; the second flow was (1.7 mmol/gdw/h, v = ~8) to pyruvate via pyruvate-ferredoxin oxidoreductase; and the third flow was into biomass production (e.g., synthesis of leucine and

fatty acids). The genome annotation indicated that some key enzymes in gluconeogenesis were missing (EC 4.1.2.13, fructose-bisphosphate aldolase; EC2.7.2.3, phosphoglycerate kinase; EC5.4.2.4, bisphosphoglycerate synthase; i.e., no reactions for glycerate-3P → glycerate-1,3-$P_2$ and glyceraldehyde-3P → β-D-fructose-1,6-$P_2$). However, the tracer experiments indicated that gluconeogenesis is actually complete, and the total flux was 0.5 mmol/gdw/h (v = ~2.5). The pentose phosphate pathway (PPP) is mainly used for biosynthesis when acetate is used as the sole carbon source. Although there are several alternative pathways to make C5P (ribose-5-phosphate, i.e., precursors of histidine and nucleotides), the model indicates that the major carbon flow to PPP is via the oxidative branch G6P → 6PG → C5P, which generates NADPH. In general, the isotopomer model gave results consistent with the previous predictions from a constraints-based model for a closely related species, *Geobacter sulfurreducens* (22). However, the presence of phospho*enol*pyruvate carboxykinase was not predicted by the constraints-based model, but was found using the isotopomer model.

***Characterization of GS-15 metabolism under $Fe^{3+}$ reduction conditions.*** Previous reports indicate that *Geobacter* possesses two acetyl-CoA production routes (via acetyl-CoA transferase or acetate kinase/P-transacetylase) to secure sufficient flux for growth, whereas other acetate-degrading anaerobic bacteria often use one pathway for acetyl-CoA formation. This study also indicated the flexibility of central metabolism in other carbon utilization routes. For example, pyruvate carboxylase activity was present (0.7 mmol/gdw/h, v=~3.6) — this is an alternative pathway to feed carbon into the TCA cycle by consuming ATP. Second, two carbon flows lead to phospho*enol*pyruvate synthesis via pyruvate kinase / phospho*enol*pyruvate synthase (~0.4 mmol/gdw/h, v = ~1.8) or phospho*enol*pyruvate carboxykinase (~0.1 mmol/gdw/h, v = ~0.6). The presence of redundant pathways may stabilize cellular metabolism

under conditions of environmental uncertainty (33). Meanwhile, the absence of the glyoxylate shunt (this pathway has not been annotated) was confirmed by the isotopomer analysis. On the other hand, the $NADP^+$-dependent malic enzyme, which is inhibited by the presence of acetyl-CoA (13) and whose corresponding gene was annotated in the genome, had no flux. These results are consistent with the predictions from the genome-scale, constraints-based model (22). With respect to energy production, zero flux through the glyoxylate shunt and malic enzyme maximizes the total carbon flow through the oxidative TCA cycle and thus produces the most reducing power (NADH).

In general, decarboxylation reactions, such as the oxidative reactions in the pentose phosphate pathway and the TCA cycle, are frequently irreversible (30). However, the model predicted extremely high reversibility (exch = 0.99) in the reaction that convert oxoglutarate to succinate compared to those in other microorganisms (41). This reaction contains two steps and is usually catalyzed by the enzymes oxoglutarate oxidoreductase (oxoglutarate → succinyl-CoA, $\Delta G^{\Theta}$ = -33.5kJ/mol) and succinyl-CoA synthetase (succinyl-CoA → succinate, $\Delta G^{\Theta}$ = -2.9 kJ/mol) (25). The free energy of both steps indicates a positive driving force for converting oxoglutarate to succinate. However, the succinyl-CoA synthetase activity is absent in *Geobacter metallireducens*, and acetyl-CoA transferase instead is used to complete the reaction: succinyl-CoA (+acetate) → succinate (+acetyl-CoA) (13). The reason for the very high reversibility between oxoglutarate and succinate is likely that the accumulation of acetyl-CoA forces the reaction in the reverse direction, and thus inhibits the rate of carbon metabolism through TCA cycle. This may explain the slow growth of *Geobacter metallireducens* under iron-reducing conditions, even though the organism can use the complete TCA cycle to oxidize carbon substrates similar to other aerobic bacteria.

Growth of *G. metallireducens* while oxidizing acetate requires incorporation of $CO_2$ into biomass (acetyl-CoA + $CO_2$ → pyruvate and pyruvate + $CO_2$ → oxaloacetate), and therefore our model evaluated the fate of the labeled $^{13}C$ of carbon dioxide. Both experiments with [1-$^{13}C$]acetate and [2-$^{13}C$]acetate indicated that the [$^{13}C$]$CO_2$ in the medium was below 3% of total $CO_2$ (Table 1). This is consistent with the fact that the labeled $^{13}CO_2$ produced from acetate oxidization is negligible compared to the $^{12}CO_2$ from the headspace gases ($N_2$-$CO_2$). The experiment performed with [1-$^{13}C$]acetate introduced very little $^{13}C$ (<3%) into the C1 pool (5,10-Me-THF), while most of the C1 pool was labeled (82%) in the [2-$^{13}C$]acetate experiments. This result confirms that C1 metabolism is mainly via the serine pathway, i.e., serine is converted to glycine and a C1-unit before being incorporated into protein. The carbon transition routes are $^{*}CH_3COOH$ → $^{*}CH_3COCOOH$ → $^{*}CH_2(OH)CH(NH_2)COOH$ → $CH_2NH_2COOH$ + $^{*}$C1 pool.

In conclusion, this study demonstrates $^{13}C$ metabolic flux analysis as a tool for verifying genome annotation, characterizing the physiological state of microorganisms, and mapping the central metabolism in anaerobic bacteria. The results from our technique provide valuable complementary information to genome-based modeling approaches resulting in a comprehensive understanding of central carbon metabolism in microorganisms. Our study indicates that *G. metallireducens* strain GS-15 utilizes the complete TCA cycle to oxidize acetate to $CO_2$ while reducing soluble Fe(III)-NTA. A futile pathway (pyruvate → oxaloacetate → PEP) is also evident by isotopomer data. Although the annotated genome indicates the absence of a few key enzymes in *gluconeogenesis* and some amino acid synthesis pathways, our $^{13}C$-tracer experiments demonstrate that those pathways are actually complete and *G. metalliredicens* may contain some undocumented metabolic routes, e.g., an unusual isoleucine biosynthesis pathway

possibly via citramalate as the intermediate is suggested by isotopic data. In combination with physiological data on the environmentally relevant microbe *Geobacter metalliredicens*, this study helps our understanding of carbon assimilation in the survival of such organisms in the environment.

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## Figure Captions

**Figure 1.** *Geobacter metallireducens* GS-15 growth kinetics in minimal medium: □, total cell number; ♦, $Fe^{2+}$ concentration; ▲, acetate concentration.

**Figure 2.** Metabolic flux distribution in *Geobacter metallireducens* GS-15 under $Fe^{3+}$ reduction conditions. The upper number indicates flux based on [1-$^{13}$C]acetate experiments, and the lower number indicates flux based on [2-$^{13}$C]acetate experiments. The acetate uptake rate was 21 mmol/gdw/h. The data in brackets are the exchange coefficients. The dotted arrows indicate the absence of an annotated gene for the step. Abbreviations: 6PG, 6-phosphogluconate; ACoA, acetyl-coenzyme A; C1, 5,10-Me-THF; C5P, ribose-5-phosphate (or ribulose-5-phosphate or xylulose-5-phosphate); CIT, citrate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; ICT, isocitrate; MAL, malate; OAA, oxaloacetate; OXO, 2-oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; PYR, pyruvate. S7P, sedoheptulose-7-phosphate; SUC, succinate; and T3P, triose-3-phosphate.

**Figure 3.** Model quality test. ♦, glutamate data; □, aspartic acid data; ◊, alanine and leucine data; Δ, serine and glycine data; ×, histidine data; ○, phenyalanine data; +, isoleucine (isoleucine data were not used as constraints for the model calculation). The absolute GC-MS measurement errors were based on the information in Table 1.

**Figure 1.**

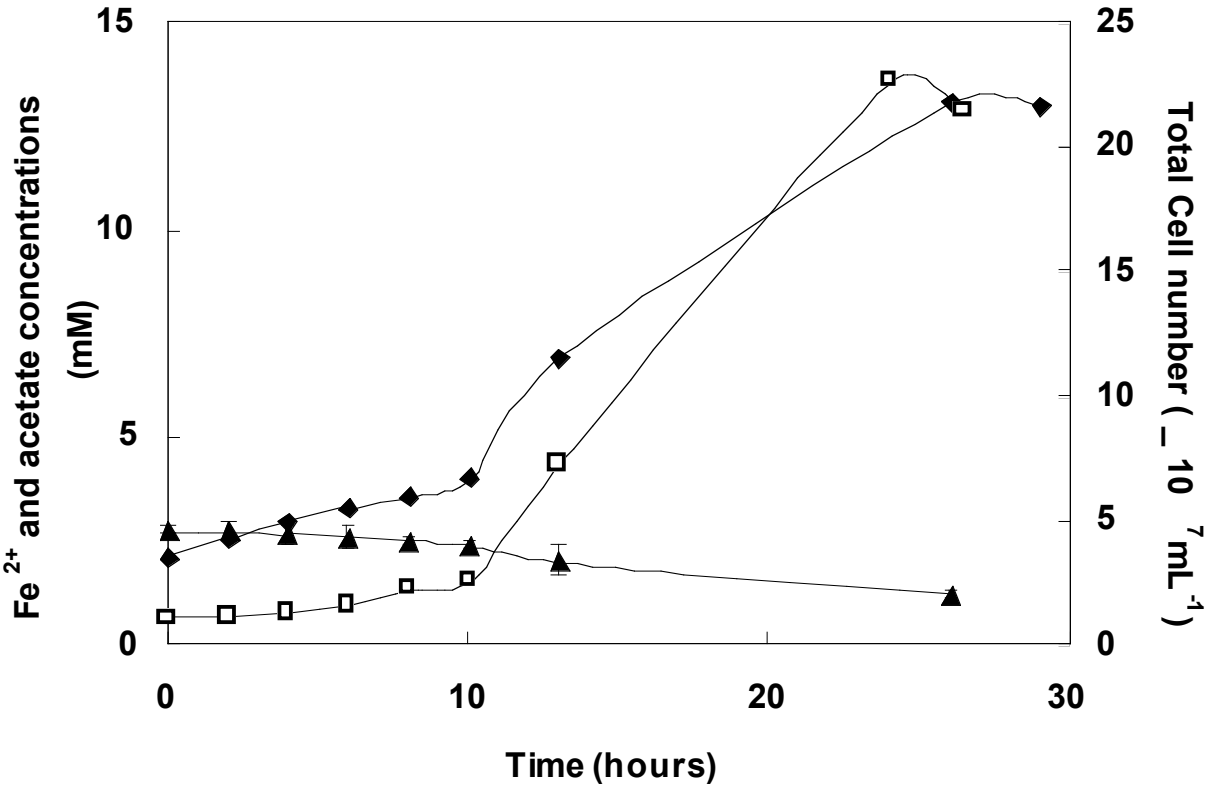
15
10
5
0
25
20
15
10
5
0
0
10
20
30
Time (hours)
Fe 2+ and acetate concentrations (mM)
Total Cell number ( _ 10 7 mL -1 )

**Figure 2.**

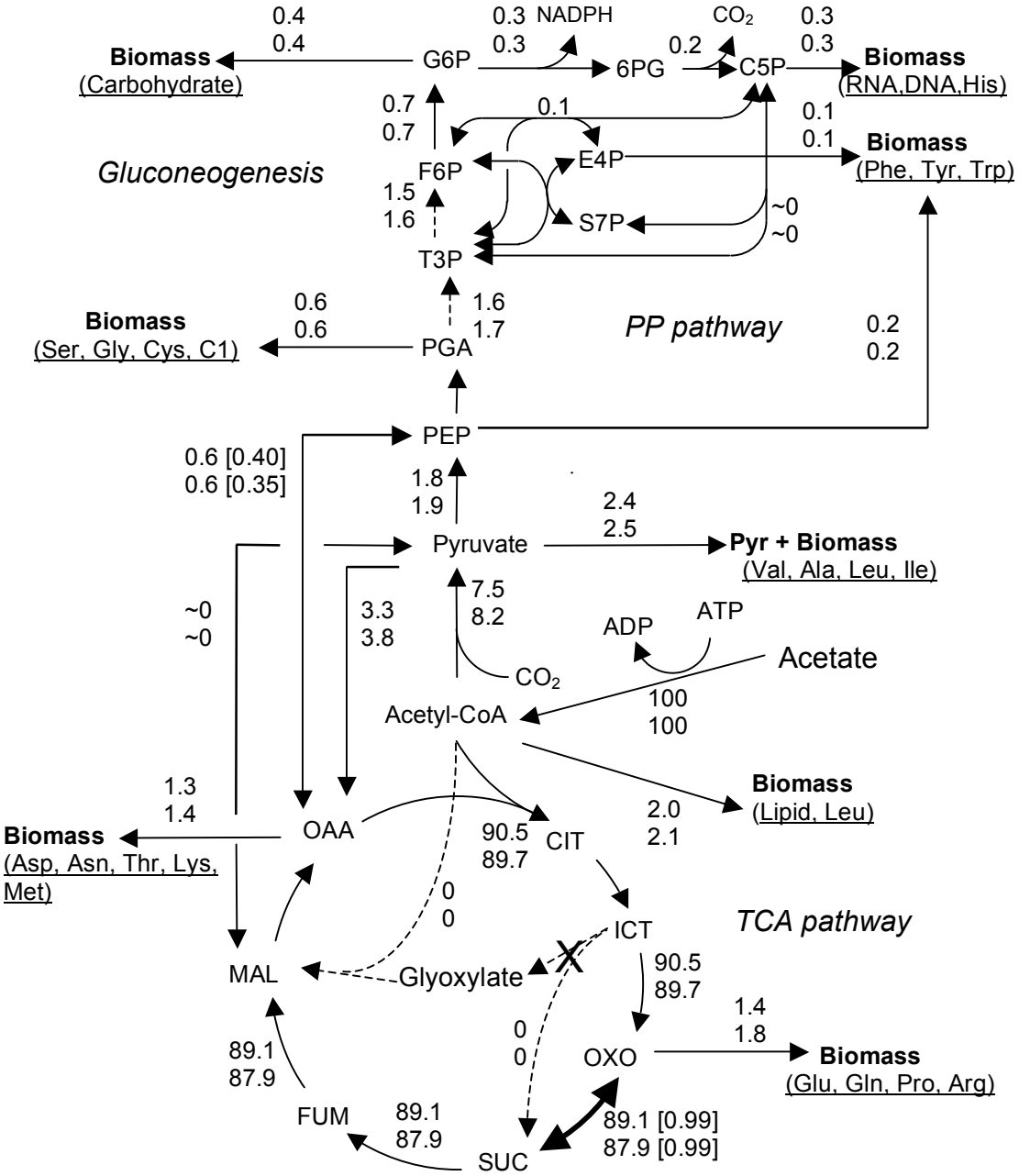
0.4
0.4
Biomass
(Carbohydrate)
G6P
0.3 NADPH
0.3
6PG
CO2
0.2
C5P
0.3
0.3
Biomass
(RNA,DNA,His)
0.7
0.7
0.1
0.1
0.1
Biomass
(Phe, Tyr, Trp)
Gluconeogenesis
F6P
E4P
1.5
1.6
S7P
~0
~0
T3P
0.6
0.6
Biomass
(Ser, Gly, Cys, C1)
PGA
1.6
1.7
PP pathway
0.2
0.2
PEP
0.6 [0.40]
0.6 [0.35]
1.8
1.9
2.4
2.5
Pyruvate
Pyr + Biomass
(Val, Ala, Leu, Ile)
~0
~0
3.3
3.8
7.5
8.2
ADP
ATP
Acetate
CO2
Acetyl-CoA
100
100
1.3
1.4
Biomass
(Asp, Asn, Thr, Lys,
Met)
OAA
90.5
89.7
CIT
2.0
2.1
Biomass
(Lipid, Leu)
0
0
ICT
TCA pathway
MAL
Glyoxylate
90.5
89.7
1.4
1.8
0
0
OXO
Biomass
(Glu, Gln, Pro, Arg)
89.1
87.9
FUM
89.1
87.9
SUC
89.1 [0.99]
87.9 [0.99]

**Figure 3.**

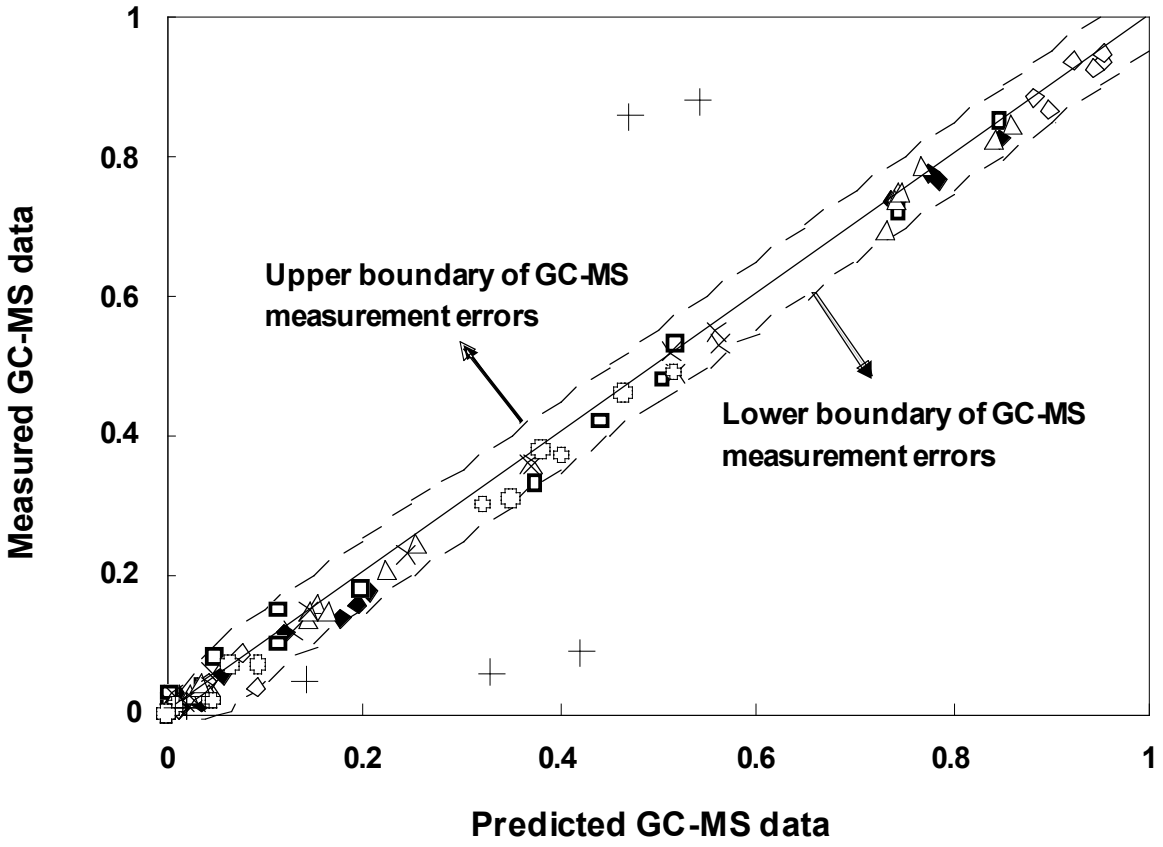
Measured GC-MS data
Predicted GC-MS data
Upper boundary of GC-MS measurement errors
Lower boundary of GC-MS measurement errors
0
0.2
0.4
0.6
0.8
1

**Table 1.** Measured fragment mass distributions for $^{13}$C-labeled amino acids from *Geobacter metallireducens* GS-15 hydrolysates[1].

| Amino acids (Precursors) | Fragment | [1-$^{13}$C]acetate culture | | | |
|---|---|---|---|---|---|
| | | $M_0$ | $M_1$ | $M_2$ | $M_3$ |
| Glycine (PEP)[2] | $(M57)^+$ | 0.15±0.02 | 0.85±0.03 | | |
| | $(M85)^+$ | 0.26±0.02 | 0.74±0.03 | | |
| Serine (PEP) | $(M57)^+$ | 0.14±0.02 | 0.83±0.03 | 0.03±0 | |
| | $(M159)^+$ | 0.25±0.02 | 0.75±0.03 | 0.0±0 | |
| Alanine (pyruvate) | $(M57)^+$ | 0.05±0.02 | 0.94±0.02 | 0.01±0 | |
| | $(M159)^+$ | 0.05±0.02 | 0.95±0.01 | 0±0 | |
| Leucine (pyruvate + acetyl CoA) | $(M159)^+$ | 0.01±0 | 0.09±0.02 | 0.87±0.02 | 0.02±0 |
| Isoleucine (OAA + pyruvate) | $(M159)^+$ | 0.01±0 | 0.09±0.01 | 0.88±0.02 | 0.01±0.01 |
| Glutamate (OXO) | $(M57)^+$ | 0.02±0 | 0.77±0.03 | 0.14±0.02 | 0.03±0.01 |
| | $(M159)^+$ | 0.02±0.01 | 0.83±0.02 | 0.12±0.02 | 0.02±0.01 |
| Asparate (OAA) | $(M57)^+$ | 0.04±0.02 | 0.85±0.03 | 0.10±0.02 | 0.01±0 |
| | $(M159)^+$ | 0.42±0.03 | 0.53±0.03 | 0.03±0.01 | 0.02±0.01 |
| Histidine (C5P) | $(M57)^+$ | 0.04±0.01 | 0.36±0.02 | 0.55±0.02 | 0.02±0 |
| | $(M159)^+$ | 0.06±0.02 | 0.36±0.02 | 0.53±0.02 | 0.02±0 |
| Phenylalanine (PEP+E4P) | $(M57)^+$ | 0.01±0 | 0.07±0.01 | 0.29±0.04 | 0.49±0.05 |
| | $(M159)^+$ | 0.01±0 | 0.07±0.01 | 0.31±0.04 | 0.46±0.05 |
| Predicted $CO_2$ | | 0.01 | | | |
| Predicted C1pool | | 0.02 | | | |

**Table 1 (Continued)**

| Amino acids (Precursors) | Fragment | [2-$^{13}$C]acetate culture | | | |
|---|---|---|---|---|---|
| | | $M_0$ | $M_1$ | $M_2$ | $M_3$ |
| Glycine (PEP)[2] | $(M57)^+$ | 0.75±0.03 | 0.15±0.02 | 0.10±0.02 | |
| | $(M85)^+$ | 0.79±0.03 | 0.21±0.02 | | |
| Serine (PEP) | $(M57)^+$ | 0.04±0.01 | 0.70±0.03 | 0.16±0.02 | |
| | $(M159)^+$ | 0.05±0.02 | 0.79±0.03 | 0.16±0.02 | |
| Alanine (pyruvate) | $(M57)^+$ | 0.03±0.01 | 0.93±0.01 | 0.02±0.01 | |
| | $(M159)^+$ | 0.03±0 | 0.93±0.01 | 0.04±0.01 | |
| Leucine (pyruvate + acetyl CoA) | $(M159)^+$ | 0.01±0 | 0.01±0 | 0.04±0.01 | 0.89±0.02 |
| Isoleucine (OAA + pyruvate) | $(M159)^+$ | 0.01±0 | 0.01±0.01 | 0.05±0.01 | 0.87±0.01 |
| Glutamate (OXO) | $(M57)^+$ | 0.02±0 | 0.01±0 | 0.16±0.01 | 0.74±0.02 |
| | $(M159)^+$ | 0.02±0 | 0.02±0 | 0.18±0.02 | 0.78±0.03 |
| Asparate (OAA) | $(M57)^+$ | 0.02±0.01 | 0.08±0.01 | 0.18±0.02 | 0.72±0.03 |
| | $(M159)^+$ | 0.03±0.02 | 0.15±0.02 | 0.48±0.02 | 0.33±0.02 |
| Histidine (C5P) | $(M57)^+$ | 0.02±0 | 0.01±0 | 0.15±0.02 | 0.52±0.03 |
| | $(M159)^+$ | 0.03±0.01 | 0.12±0.01 | 0.49±0.02 | 0.23±0.02 |
| Phenylalanine (PEP+E4P) | $(M57)^+$ | 0.01±0 | 0±0 | 0.02±0 | 0.38±0.03 |
| | $(M159)^+$ | 0.01±0 | 0±0 | 0.02±0 | 0.36±0.02 |
| Predicted $CO_2$ | | 0.03 | | | |
| Predicted C1pool | | 0.82 | | | |

1. $^{13}$C-Labeled biomass was sampled in the middle log phase. The standard deviations for GC-MS measurement were based on the triplicate experiments (n=3).
2. Glycine fragmentation $(M159)^+$ was not observed. $(M85)^+$ (Loss of carboxyl group) was used instead.